ETHANOL IN BIOLOGICAL SPECIMENS BY DUAL HEADSPACE GAS CHROMATOGRAPHY (HS/GC)

PRINCIPLE

Volatile compounds are analyzed in biological fluids by gas chromatography using N-propyl alcohol as the internal standard. Water containing an internal standard is added simultaneously with the biological sample as it is sampled using an automatic diluter. It is then sealed in a headspace sample vial prior to analysis. Volatile sample components are extracted from the non-volatile sample components by heating, pressurizing the vial and then sampling from the equilibrated gas phase above the sample phase. One milliliter of this gas phase mixture is injected onto a column, which splits into 2 different gas chromatographic columns. The volatile compounds are separated based on their respective molecular weights and polarities and detected with a flame ionization detector. The identification of ethanol and other volatile compounds is made by comparing the relative retention times of the unknown to the retention time of an internal standard. The ratio of sample peak area to internal standard peak area is compared to the calibration curve to provide a quantitation of volatile compounds in the sample.

By using 2 different columns that cause the volatiles to separate in different but known ways, a more specific identification is possible. The possibility of an interfering or co-eluting peak is also considerably reduced since it is unlikely to elute on both columns at the same retention time.

Samples are screened using the Volatiles Screen analytical method on the HS/GC. Positive samples are then quantified using the Ethanol Quantitation analytical method on the HS/GC.

SPECIMEN

1. BLOOD: Use blood specimens collected in gray top evacuated tubes (containing 100 mg sodium fluoride and 20 mg potassium oxalate). Blood specimens collected in other containers may be analyzed. The optimum sample size is 2 mL or greater. Specimens containing less than 2 mL may be analyzed.
2. URINE: Use urine specimens collected in urine collection bottles. Urine specimens collected in other containers may be analyzed. The optimum specimen size is 2 mL or greater. Specimens containing less than 2 mL may be analyzed.
3. VITREOUS FLUID: Use vitreous fluid specimens collected in red top tubes. Vitreous fluid specimens collected in other containers may be analyzed. The optimum specimen size is 2 mL or greater. Specimens containing less than 2 mL may be analyzed.
4. Keep specimens at 2°C to 8°C until analyzed; bring the specimens to room temperature before analysis.

5. If a specimen contains an insufficient sample for analysis, the submitting agency is notified on the final report.

SAFETY PRECAUTIONS

1. Wear safety glasses, laboratory coats, and gloves when handling reagents, samples, and controls.
2. Sample aliquoting procedures must be performed under a biosafety hood.
3. Processed headspace vials are both heated and pressurized; take caution when removing from the autosampler, as they may burst upon impact.

REAGENTS AND SUPPLIES

1. N-propyl alcohol (high purity)[1,2,3]
2. Commercially prepared Ethanol Calibrators (0.01, 0.02, 0.05, 0.08, 0.20, 0.40, 0.50 g/dL)
3. Commercially prepared Whole Blood Reference Control (targeted at 0.05 g/dL and 0.20 g/dL per manufacturer)
4. Commercially prepared Serum Interference Control (targeted at 0.15 g/dL Ethanol, 0.08 g/dL Acetone, 0.08 g/dL Isopropanol, and 0.04 g/dL Methanol per manufacturer)
5. 2L volumetric flask
6. 5 mL volumetric pipettes
7. Adjustable Pipetter 10-100 µL
8. Agilent 10 mL headspace vials or equivalent
9. 20 mm butyl septa
10. 20 mm aluminum seals
11. Headspace vial racks
12. Automatic diluter
13. Tube rocker
14. Homogenizer
15. Agilent Technologies 7890A Gas Chromatograph with dual flame ionization detector
16. Agilent Technologies G1888 Headspace Sampler

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[1] Highly Flammable – avoid heat or flame.
[2] Irritant – avoid contact with skin, eyes, etc. – avoid inhaling fumes.
[3] Health – may be harmful if swallowed, inhaled, or absorbed through skin.

17. Agilent J & W DB-ALC1 column 30 m length 0.32 mm diameter
18. Agilent J & W DB-ALC2 column 30 m length 0.32 mm diameter
19. Chemstation™ software

STANDARDS PREPARATION

Label all reagents with reagent name, tracking number, preparation date, preparer's initials, and expiration date. Store in a ground glass stoppered reagent bottle. Record the standard reagent information in LIMS.

Ethanol Working Standard Preparation:

After opening the ethanol standard ampoule, transfer the solution to its individual amber glass vial and cap with a screw cap. Standards are good for 2 weeks from the time they are opened. Store vials in refrigerator at 2°C to 8°C when not in use.

INTERNAL STANDARD PREPARATION

N-propyl Alcohol Internal Standard (IS) Preparation:

1. Add 200 µL N-propyl Alcohol to 2 L ultrapure water and mix well. Add 250 mg of sodium fluoride to prevent mold growth. Label reagent bottle with internal standard name, lot number, analyst's initials, and tracking number. Internal standard solution is good for 1 year from the date of preparation and can be stored at room temperature.
2. New lots of Internal Standard must be validated prior to use in casework.
3. Record the preparation and verification results in LIMS and on hardcopy using the required form. File the original form with the verification batch and submit a copy to the QA Manager.

CONTROL PREPARATION

All controls must be prepared from a separate stock source than the standards. Separate vendors and/or lot numbers are sufficient to fill this requirement.

1. Whole Blood Reference Control:

   Prepared by manufacturer. Upon receipt, store at -10°C to -20°C until use. After opening, store in the refrigerator at 2°C to 8°C when not in use; expiration is 21 days from the time it is opened.

2. Serum Interference Control (contains ethanol, methanol, acetone, isopropanol):

   Prepare according to manufacturer instructions. Store in the refrigerator at 2°C to 8°C; expiration is 30 days from the time it is prepared.

CALIBRATION PROCEDURE

1. Calibrators consist of seven levels: 0.01, 0.02, 0.05, 0.08, 0.20, 0.40, 0.50 g/dL.

2. Calibrate the gas chromatograph prior to use with calibrators in order of increasing concentration. The same calibration curve is used for the screen and quantitation.
3. Values must fall within ± 10% of the target values before new calibrator lots can be introduced.

QUALITY CONTROL PROCEDURE

1. Quality controls samples consist of:
    - 1.1. Low and High Whole Blood Reference Control is used to evaluate the accuracy of the ethanol result.
    - 1.2. Serum Interference Control (contains ethanol, methanol, acetone, isopropanol) is used to evaluate the presence of methanol, acetone, and isopropanol.
2. The controls are used for the screen and quantitation.
3. Before a new Low or High Whole Blood Reference Control lot can be introduced, it must be verified by running 7 replicates of the control. The target for the new Low or High control is set using the mean value of the 7 replicates.
4. Record Whole Blood Reference Control results in LIMS.
5. Values for the new Low or High control must fall within ± 10% of the target values.
6. Responses for each of the 4 peaks in the Serum Interference Control must be greater than the screen lower reporting limits before the new lot can be introduced.

SAMPLE PREPARATION PROCEDURE

1. Bring calibrators, controls, and samples to room temperature.
2. Mix blood samples for at least 3 minutes on a tube rocker. Clotted samples should be homogenized prior to sampling and this step should be indicated in the batch worksheet.
3. Use the automatic diluter to dispense 1.4 mL of internal standard mixture and 0.05 mL of calibrators, controls, and samples into a headspace vial. Cap each headspace vial immediately after dispensing.
4. An internal standard blank is prepared by placing the internal standard mix into a sample vial.
5. Seal the headspace cap onto the specimen by crimping each vial.
6. Using the Chemstation™ software, enter the analytical sequence in the following order for screens:

Calibrators (in increasing order)

Internal Standard Blank

Serum Reference Control

Low Whole Blood Control

High Whole Blood Control

≤ 20 screen injections

Low Whole Blood Control

High Whole Blood Control

≤ 20 screen injections

Low Whole Blood Control

High Whole Blood Control, etc.

7. Load the vials onto the autosampler tray according to the analytical sequence and analyze the samples with the Chemstation™ Ethanol program. Typical HS/GC conditions are included in the Instrument Log binder.

8. After the batch has been authorized by a technical review, prepare new aliquots of the whole blood control and the samples positive for ethanol as described in steps 1-5. Samples positive for methanol, acetone, and isopropanol will be quantitated according to the Volatiles Quantitation SOP.

9. The sequence order for quantitations continues on the batch in the following order:

Low Whole Blood Control

High Whole Blood Control

≤ 20 quantitation injections

Low Whole Blood Control

High Whole Blood Control

≤ 20 quantitation injections

Low Whole Blood Control

High Whole Blood Control, etc.

ACCEPTANCE CRITERIA AND CALCULATIONS

1. A calibration curve is derived by comparison between the ratios of the calibrator ethanol peak areas to their respective internal standard peak areas. The ratio of sample peak area to internal standard peak area is compared to the calibration curve to provide a quantitation of compounds in the sample. The Chemstation™ software calculates a "least squares" line.

1.1. The calibrator coefficient (r^2) must be $\geq$ 0.99.

1.2. A minimum of 5 calibrators is needed to construct the calibration curve.

1.3. Calibration points may be removed from the curve in order to improve the linearity of the curve. If the highest or the lowest calibration points are removed the the reporting limit must be adjusted accordingly. No more than 2 calibration points may be removed. Calibration points removed from the curve must be noted under Batch Comment in LIMS.

2. The dual HS/GC generates 2 values for each injection because it uses 2 separate columns.

2.1. Internal Standard blank: The mean of the blank results must be less than the lower reporting limits for ethanol, methanol, acetone, and isopropanol.

2.2. Low and High Whole Blood Control: The mean of the 2 values for Low Whole Blood and the mean of the 2 values for High Whole Blood Control must be within ± 10% of the in house target concentration for their particular lot numbers.

2.3. Serum Interference Control: The mean of the 2 values for Serum Interference Control must fall within ± 10% of the manufacturer target concentration for ethanol and responses must be greater than the lower reporting limits for methanol, acetone, and isopropanol.

2.4. Samples: The mean of the sample results is used to calculate the result as described below.

2.4.1. Screen results for Ethanol:

2.4.1.1. For any LE samples > LE lower reporting limit, report the mean of 2 values generated from the Volatile Screen. The 2 values must fall within ± 5% of their mean.

2.4.1.2. For any ME samples > ME lower reporting limit, report the mean of the 2 values generated from the Volatile Screen. The 2 values must fall within ± 5% of their mean.

2.4.2. Screen results for Methanol, Acetone, and Isopropanol:

2.4.2.1. For all samples with responses for Methanol, Acetone, and Isopropanol that are greater than the corresponding screen lower reporting limits, report as “Positive”.

2.4.2.2. These samples will be quantitated according to the Volatiles Quantitation SOP.

2.4.3. Quantitation results for Ethanol: All values are truncated after the 3rd decimal as they are entered on LIMS. The mean of the 2 values generated from Ethanol Quantitation must fall within ± 5% of their mean.

2.4.4. The mean of the Volatile Screen result and the mean of the Ethanol Quantitation result must fall within 10% of each other. If these requirements are met, the lowest value of the 4 values generated is reported to the 2nd decimal place.

2.4.5. Any samples with a result greater than the highest calibrator may be re-analyzed using a smaller aliquot of the sample, so the result falls within the calibration concentration range. Alternatively, the sample result may be reported as having a concentration greater than the value of the highest calibrator. Dilutions should be prepared with the appropriate matrix and documented on the analytical results.

3. Both the instrument and LIMS calculations are verified annually as described in the Calculation Verification SOP.

REPORTING RESULTS

1. Calibration results are printed on hardcopy.
2. Blank control and Serum control results are printed and entered to the third decimal place on LIMS. No additional blank or serum controls are included for quantitation.
3. Low and High Whole Blood Control results:
   - 3.1. For screens: results are printed and entered to the third decimal place on LIMS.
   - 3.2. For quantitations: results are printed and entered to the third decimal place on LIMS.
4. Sample results:
   - 4.1. For screens: results are printed and entered to the third decimal place on LIMS.
   - 4.2. For quantitations: results are printed and entered to the third decimal place on LIMS.
5. Reagent information and batch QC information are entered on LIMS. Notes relating to batch QC information are included under “Criteria Exceptions”. Other notes relating to the batch are included under “Batch Comment.”

LIMITATIONS OF PROCEDURE

1. For Ethanol:
   - 1.1. The LE reporting limit = 0.01 g/dL.
   - 1.2. The ME reporting limit = 0.02 g/dL.
   - 1.3. The upper reporting limits for both LE and ME cases = 0.50 g/dL.
2. For Methanol, Acetone, and Isopropanol: the screen lower reporting limits are:

Analyte	Headspace 1 Response	Headspace 2 Response
Methanol	10	5
Isopropanol	50	15
Acetone	100	30

PROCEDURE NOTES

1. There is one sample transfer recorded for the screen and when applicable, a separate sample transfer recorded for quantitation.
2. Any sample that overloads the detector may be diluted and reanalyzed.
3. Any sample with large extraneous peaks should be evaluated for the presence of other volatile compounds and/or interferences. For some common interferences, see Appendix A.

REFERENCES

1. Agilent Chemstation help index, topic: Calibration curves.
2. Roger L. Firor and Chin-Kai Meng, "Static Headspace Blood Alcohol Analysis with the G1888 Network Headspace Sampler".
3. Restek Corp.'s "A Technical Guide for Static Headspace Analysis Using GC".
4. Matthew T. Barnhill, Jr., Donald Herbert, and David J. Wells, Jr., "Comparison of Hospital Laboratory Serum Alcohol Levels Obtained by Enzymatic Method with Whole Blood Levels Forensically Determined by Gas Chromatography" Journal of Analytical Toxicology Vol. 31 2007
5. Butala, Steven J.M., PhD, "Estimation of Bureau of Toxicology Laboratory Error for Blood Alcohol Utilizing Direct Injection GC/FID: Addendum: Estimation of Bureau of Toxicology Laboratory Error for Blood Alcohol Utilizing Headspace GC/FID" Bureau of Environmental Chemistry, Utah Department of Health.

APPENDIX A: Relative Retention Times (RRTs) for Miscellaneous Volatile Compounds

The following table provides a summary of RRTs of other volatile compounds on the two different columns of the HS/GC. With only one exception (chloroform), all tested interferences caused conflicts in only 1 column and may be compensated for by using data from the other column.

The minor peaks for chloroform are the only known interferences for both columns, and this occurs only when the chloroform has broken down. When fresh chloroform is present, no minor peaks were observed. If the main chloroform peak is present then it *may* contribute to any ethanol seen.

Elution Order DB-Alc1				Elution Order DB-Alc2		
#	Compound	RRT db-alc1		#	Compound	RRT db-alc2
1	Methanol	0.493		1	Acetaldehyde	0.435
2	Formaldehyde	0.501/0.833		2	Methanol	0.464
3	Acetaldehyde	0.542		3	Formaldehyde	0.474 / 0.580
4	Ethanol	0.616		4	Ethyl ether	0.516
5	Ethyl ether	0.736		5	Ethanol	0.584
6	Isopropyl alcohol	0.746		6	Acetone	0.642
7	Methylene chloride	0.826		7	Hexane	0.666 **/m**
8	Acetone	0.898		8	Isopropyl alcohol	0.683
9	Acetonitrile	0.907		9	Methylene chloride	0.697
10	N-propyl alcohol (IS)	1.000		10	Acetonitrile	0.794
11	Hexane	1.146 **/m**		11	N-propyl alcohol (IS)	1.00
12	Chloroform	1.285 **/m**		12	Ethyl acetate	1.05 **/m**
13	Iso-butyl alcohol	1.550		13	Heptane	1.152

14	Ethyl acetate	1.680		14	Chloroform	1.183 **/m**
15	n-Butyl alcohol	1.985 **/m**		15	Iso-butyl alcohol	1.590
16	Heptane	2.280		16	n-Butyl alcohol	2.115 **/m**
17	Iso-amyl Alcohol	3.530 **/m**		17	Toluene	2.880 **/m**
18	Toluene	4.215		18	Iso-amyl Alcohol	3.760 / 3.830 **/m**
19	m-Xylene	9.750+		19	m-Xylene	6.824

m = multiple minor peaks

AUTHORIZATION

QA Manager Approval: Signature: Date: 08/14/19

Lab Director Approval: Signature: Date: 8/14/19